Regulation of Immune Responses by Vitamin D Receptor Ligands

Luciano Adorini

BioXell, Milan, Italy

Address correspondence to Luciano Adorini, BioXell, Via Olgettina 58, I-20132 Milano, Italy. Tel. +39-02-2884816, Fax: +39-02-2153203, e-mail: Luciano.Adorini@bioxell.com

I. INTRODUCTION

The *raison d' etre* of the immune system is to maintain the biological integrity of the individual. This is accomplished by two layers of immune responses, innate and adaptive. Innate immune responses can be induced in virtually any cell, but they are primarily mediated by specialized cell types, such as macrophages and natural killer cells. Innate immunity is characterized by rapid, local responses, largely based on the production of pro-inflammatory mediators, in particular cytokines, chemokines, and reactive oxygen species. This is triggered by recognition of stereotyped patterns conserved in infectious microorganisms via toll-like receptors (TLRs), surface molecules able to recognize distinct structural components of pathogens [1]. Activation of signal transduction pathways by TLRs leads to upregulation of different genes that operate in host defence, including co-stimulatory molecules, cytokines and chemokines [2].

Shortly afterwards, adaptive immunity can be induced. Adaptive immune responses are induced by cells specialized in antigen processing and presentation, in particular dendritic cells (DCs), and are mediated by cells specialized in antigen recognition, carried out by T and B lymphocytes. Adaptive immune responses are primarily orchestrated by $CD4^+$ T lymphocytes. To select lymphocytes able to respond to foreign molecules while remaining tolerant to self components, the strategy of the immune system has been to generate a vast repertoire of antigen-specific receptors, distribute it clonally in different lymphocytes, and then eliminate cells capable of recognizing with high affinity self components while permitting the differentiation of T cells potentially able to recognize foreign antigens. However complex as to the mechanisms utilized, the basis for tolerance to self components is relatively simple. First, T cells expressing high-affinity receptors for self antigens can be physically eliminated during thymic development via clonal deletion. Although this is a primary mechanism of self tolerance, it does not completely eliminate T cells specific for self antigens. Self-reactive T cells that have been exported to the periphery can then be functionally inactivated upon antigen recognition in the absence of appropriate costimulatory signals, a

process denominated clonal anergy. Finally, peripheral self-reactive T cells can be suppressed by other T cells. In reality, tolerance is truly redundant, and both deletional and non-deletional mechanisms operate in the thymus and in the periphery [3].

A: Selective Immunointervention

Failure of tolerance mechanisms may lead to autoimmune diseases and to other immune-mediated pathologies. The progress in understanding the mechanisms of T cell activation and inactivation is currently being translated into strategies able to induce selective immunosuppression to treat different pathological situations, notably autoimmune diseases, as well as allergies and allograft rejection. The medical need for selective immunosuppression is very high, as the available immunosuppressive drugs are substantially inadequate because of limited efficacy, modest selectivity, and considerable toxicity [4].

Key attack points for selective immunointervention have been identified: modulation of antigen recognition, costimulation blockade, induction of regulatory cells, deviation to non-pathogenic or protective responses, neutralization of proinflammatory cytokines, induction or administration of anti-inflammatory cytokines, and modulation of leukocyte trafficking (Table 1). Thus, to selectively interfere with the activation of pathogenic T cells, immunosuppressive therapy can be primarily directed to three cellular targets: antigen-presenting cells (APCs), autoreactive T cells and suppressor/regulatory T cells, with the common goal to selectively inhibit the activation of pathogenic class II-restricted $CD4^+$ T cells [4].

In autoimmune diseases, pathogenic T cells are usually Th1 cells. $CD4^+$ T cells can be distinguished, based on their pattern of cytokine production, into three major types. Th1 cells are characterized by secretion of interferon-γ (IFN-γ), IL-2, and TNF-β, and they promote cell-mediated immunity able to eliminate intracellular pathogens. Th2 cells selectively produce IL-4 and IL-5, and are involved in the development of humoral immunity protecting against extracellular pathogens. Th0 cells,

which could either represent precursors or a terminally differentiated subset, are not restricted in their lymphokine production. A similar distinction applies to $CD8^+$ cells. The development of Th1 and Th2 cells is influenced by several factors, but three are most important: local cytokines, the avidity of ligand-TCR interaction and the non-MHC genetic polymorphism. Decisive roles in the polarization of T cells are played by IL-12 and IL-4, guiding T cell responses towards the Th1 or Th2 phenotype, respectively [3].

Different forms of immunointervention have been successfully used to prevent and sometimes treat experimental autoimmune diseases and allograft rejection (Table 1). Several of these approaches target DCs, aiming at inducing or enhancing tolerogenic properties in this APC type critically involved in modulating T cell responses. A variety of agents, both biologic and pharmacologic, have been shown to promote the intrinsic tolerogenic capacity of DCs [5,6]. Biologics include costimulation-blocking agents, such as anti-CD40L and CD152-Ig, and anti-inflammatory cytokines like IL-10 and TGF-β. Pharmacologic agents include immunosuppressive molecules such as mycophenolate mofetil, sirolimus, desoxyspergualin, corticosteroids and $1,25(OH)_2D_3$.

B. Vitamin D Receptor Ligands as Immunoregulatory Agents

$1,25(OH)_2D_3$, the activated form of vitamin D, is a secosteroid hormone that has, in addition to its central function in calcium and bone metabolism, important effects on the growth and differentiation of many cell types, and pronounced immunoregulatory properties [7-11]. The biological effects of $1,25(OH)_2D_3$ are mediated by the vitamin D receptor (VDR), a member of the superfamily of nuclear hormone receptors [12,13]. Ligand binding induces conformational changes in the VDR, which promote heterodimerization with the retinoid X receptor (RXR) and recruitment of a number of corepressor and coactivator proteins, including steroid receptor coactivator family members and a multimember coactivator complex, D receptor interacting proteins (DRIP). These coactivators induce chromatin remodeling through intrinsic histone-

modifying activities and direct recruitment of key transcription initiation components at regulated promoters. Thus, the VDR functions as a ligand-activated transcription factor that binds to specific DNA sequence elements (vitamin D responsive elements, VDRE) in vitamin D responsive genes and ultimately influences the rate of RNA polymerase II-mediated transcription [14].

The discovery of VDR expression in most cell types of the immune system [15], in particular in APCs such as macrophages [15] and DCs [16], as well as in both $CD4^+$ and $CD8^+$ T lymphocytes (reviewed in ref. [17], prompted the investigation of VDR ligands as agents able to modulate T cell responses [18]. Data accumulated in the last few years clearly demonstrate that, in addition to exerting direct effects on T cell activation, VDR ligands markedly modulate the phenotype and function of APCs, and in particular of DCs. *In vitro* and *in vivo* experiments have shown that VDR ligands induce DCs to acquire tolerogenic properties that favor the induction of regulatory rather than effector T cells. These intriguing actions of VDR ligands have been demonstrated in several experimental models and could be exploited, in principle, to treat a variety of human autoimmune diseases [19] and other immuno-mediated pathologies [7-9]. In addition, it is conceivable that $1,25(OH)_2D_3$, which is produced by macrophages [20-22], DCs [23] and T cells [21], could physiologically contribute to regulate innate and adaptive immune responses. This appealing concept, although still speculative, is mostly based on epidemiological data [7-9] and is indirectly supported by the observation that VDR deficient, compared to wild-type mice, show hypertrophy of subcutaneous lymph nodes with an increase in mature DCs [24]. Clarification of the physiological role of endogenous VDR ligands in the regulation of immune responses will likely represent a future step of development in this fruitful area of research.

II. MAJOR TARGET CELLS IN IMMUNOREGULATION BY VDR LIGANDS: DENDRITIC CELLS AND T CELLS

DCs, a highly specialized APC system critical for the initiation of $CD4^+$ T cell responses are present, in different stages of maturation, in the circulation as well as in lymphoid and non-lymphoid organs [25]. Immature DCs, such as Langerhans cells in the skin, are found in non-lymphoid tissues, where they exert a sentinel function. After antigen uptake, they migrate through the afferent lymph to T-dependent areas of secondary lymphoid organs where priming of naive T cells may occur. During migration to lymphoid organs, DCs mature into potent APCs by increasing their immunostimulatory properties while decreasing antigen-capturing capacity [26]. DCs are heterogeneous not only in terms of maturation state, but also of origin, morphology, phenotype and function [26,27]. Two distinct DC subsets were originally defined in the human blood based on the expression of CD11c, and they have been subsequently characterized as belonging to the myeloid or lymphoid lineage. Although different denominations have been used, they can be defined as myeloid (M-DCs) and plasmacytoid (P-DCs) DCs [28]. A cell population resembling human P-DCs has also been identified in the mouse [29]. M-DCs are characterized by a monocytic morphology; express myeloid markers like CD13 and CD33, the β2 integrin CD11c, the inhibitory receptor ILT1 and low levels of the IL-3 receptor α chain CD123. Conversely, P-DCs have a morphology resembling plasma cells, are devoid of myeloid markers, express high levels of CD4, CD62L and CD123. M-DCs produce high levels of IL-12, while P-DCs high levels of IFN-α [28], cytokines with clearly distinct effects on T cell activation and differentiation.

Recently, it has become clear that DCs are not only immunogenic but also tolerogenic, both intrathymically and in the periphery [30]. In particular, immature DCs have been found to have tolerogenic properties and to induce T cells with suppressive activity [31,32]. In contrast, the role of DC subsets in directing the development of T cells with a defined functional role is still unclear, although P-DCs are credited with a higher tolerogenic potential [28]. In any case, DCs expressing low levels of costimulatory molecules, either membrane-bound (e.g. CD40, CD80, CD86) or secreted, like IL-12, and high levels of

inhibitory surface molecules such as ILT3, or secreted molecules as IL-10, favour the induction of suppressor rather than effector T cells (Fig. 1).

A. Regulatory Effects of VDR Ligands in Dendritic Cells

Earlier indications for the capacity of VDR ligands to target APCs [33-35] were corroborated by their ability to inhibit the production of IL-12 [36,37], an APC-derived cytokine critical for Th1 cell development [38]. More recent work has demonstrated that $1,25(OH)_2D_3$ and its analogs have profound effects on the phenotype and function of myeloid DCs (Table 2). VDR ligands arrest the differentiation and maturation of DCs, maintaining them in an immature state, as shown by decreased expression of maturation markers and increased antigen uptake [39-44]. Collectively, studies performed either on monocyte-derived DCs from human peripheral blood or on bone-marrow derived mouse DCs, have consistently shown that *in vitro* treatment of DCs with VDR ligands leads to downregulated expression of the costimulatory molecules CD40, CD80, CD86, and to markedly decreased IL-12 and enhanced IL-10 production, resulting in inhibition of T-cell activation. The near abrogation of IL-12 production and the strongly enhanced production of IL-10 highlight the important functional effects of $1,25(OH)_2D_3$ and its analogs on DCs and are, at least in part, responsible for the induction of DCs with tolerogenic properties. In addition, DCs treated with VDR ligands upregulate the expression of ILT3, an inhibitory molecule that has been associated with tolerance induction [45].

$1,25(OH)_2D_3$ utilizes different mechanisms to regulate cytokine production by DCs. IL-12 secretion is inhibited by targeting the NF-*k*B pathway [37], via NF-*k*B proteins such as Rel-B and c-Rel [9,46]. Interestingly, antigen-exposed DCs in which Rel-B function is inhibited induce a population of antigen-specific $CD4^+$ cells that regulate immune responses in an IL-10-dependent manner [47]. Suppression of the monocyte recruiter GM-CSF is instead achieved by interaction of ligand-bound VDR monomers with

functional repressive complexes in the promoter region of the cytokine [48]. In this case, the VDR-ligand complex acts selectively on the two components required for activation of this promoter/enhancer: it competes with NFAT1 for binding to the composite site, positioning itself adjacent to *Jun-Fos* on the DNA. Co-occupancy apparently leads to an inhibitory effect on *c-Jun* transactivation function. These two VDR-mediated events effectively block the NFAT1-AP-1 activation complex, resulting in an attenuation of GM-CSF transcription [49].

The prevention of DC differentiation and maturation as well as the modulation of their activation and survival, leading to DCs with tolerogenic phenotype and function that result in T cell hyporesponsiveness, certainly play an important role in the immunoregulatory activities of VDR ligands. These effects are not limited to *in vitro* activity: $1,25(OH)_2D_3$ and its analogs can also induce DCs with tolerogenic properties *in vivo,* as demonstrated in models of allograft rejection by oral administration directly to the recipient [50] or by adoptive transfer of *in vitro*-treated DCs [24]. Tolerogenic DCs induced by a short treatment with $1,25(OH)_2D_3$ are probably responsible for the capacity of this hormone to induce $CD4^+CD25^+$ regulatory T cells that are able to mediate transplantation tolerance [50] and arrest the development of autoimmune diabetes [51]. Rag-1-dependent regulatory cells have also been implicated in the prevention of EAE induced by $1,25(OH)_2D_3$, although no effect on APCs could be demonstrated in this study [52].

B. Effects of VDR Ligands in T Cells

As reviewed above, VDR ligands modulate DC function, thus shaping T cell activation and development, but they can also have direct effects on T cells. Soon after the discovery of VDR expression in T cells [15,53], $1,25(OH)_2D_3$ was shown to inhibit antigen-induced T cell proliferation [18] and cytokine production [54]. Later studies demonstrated selective inhibition of Th1 cell development [36,55], although it was

not clarified how much of this effect could be accounted for by modulation of DC functions. Indeed, several key cytokines in T lymphocytes are direct targets for VDR ligands, in particular Th1-type cytokines such as IL-2 and IFN-γ (Table 3). $1,25(OH)_2D_3$ inhibits IL-2 secretion by impairing the transcription factor NF-AT complex formation, because the ligand-bound VDR complex binds to the distal NF-AT binding site of the human IL-2 promoter [56,57]. Another key T cell cytokine, IFN-γ, has been found directly inhibited by $1,25(OH)_2D_3$ through interaction of the ligand-bound VDR complex with a VDRE in the promoter region of the cytokine [58]. Progressive deletion analysis of the IFN-γ promoter revealed that negative regulation by $1,25(OH)_2D_3$ is also exerted at the level of an upstream region containing an enhancer element [58]. However, some *in vivo* studies have failed to support a direct effect of $1,25(OH)_2D_3$ on IFN-γ production by T cells [59].

VDR ligands are known to control the growth and differentiation of many cell types, using a variety of different mechanisms [7-9]. $1,25(OH)_2D_3$ inhibits in T cells activation-induced cell death by down-regulating the expression of CD95L, a cell surface molecule that activates apoptosis in CD95 (Fas)-expressing target cells, via repression of CD95L promoter activity through an AF-2-dependent mechanism [60]. Down-regulation of CD95L expression may have functional consequences, because CD95L costimulates the in vivo proliferation of $CD8^+$ T cells [61] and the activated CD95 (Fas) induces DC maturation and a preferential T cell polarization towards the Th1 pathway [62]. This could be one of the mechanisms VDR ligands utilize to arrest indirectly DC maturation, although they directly promote [39], rather than inhibiting, apoptosis in DCs.

$1,25(OH)_2D_3$ has been also shown to enhance the development of Th2 cells via a direct effect on naïve $CD4^+$ cells [63], and this could contribute to account for the beneficial effect of VDR ligands in the treatment of autoimmune diseases and possibly also allograft rejection. The capacity of $1,25(OH)_2D_3$ to skew T cells towards the Th2 pathway had been previously suggested [59,64], but could not be confirmed by

other studies [52,55]. A recent study has actually shown that $1,25(OH)_2D_3$ can inhibit both IFN-γ and IL-4 production in T cells [65]. The inhibition of IL-4 production in naïve T cells does not appear to result from a cell cycle block or from inhibition of Th2 transcription factor expression, but rather from a VDR-induced direct down-regulation of IL-4 transcription. It is puzzling to note that $1,25(OH)_2D_3$ can apparently up-regulate [59,63,64], down-regulate [65], or have no effect [52,55] on IL-4 production, and consequently on Th2 cell development. These disparate results may reflect the different conditions tested, but also illustrate the complex immunoregulatory pathways set in motion by $1,25(OH)_2D_3$. In addition, a novel aspect of the multiple effects of VDR ligands on T cells is provided by the induction of cells with suppressive and regulatory properties.

In conclusion, $1,25(OH)_2D_3$ *in vivo* appears primarily to inhibit Th1 cells and, under appropriate conditions, may favor a deviation to the Th2 pathway. These effects could be, in part, a consequence of direct T cell targeting by $1,25(OH)_2D_3$ and its analogs, but modulation of DC function by VDR ligands certainly plays an important role in shaping the development of T cell responses. Thus, VDR ligands can target T cells both directly and indirectly. The capacity of VDR ligands to target DCs and T cells depends on VDR expression by both cell types and on the presence of common targets in their signal transduction pathways, as exemplified by the ability of VDR ligands to down-regulate the nuclear factor NF-*k*B in DCs [9,37] and in T cells [66].

C. Enhancement of Regulatory T Cells by VDR Ligands

As discussed above, induction of DCs with tolerogenic phenotype and function plays an important role in the immunoregulatory activity of VDR ligands. Tolerogenic DCs induced by a short treatment with $1,25(OH)_2D_3$ or its analogs are likely responsible for the capacity of this hormone to induce $CD4^+CD25^+$

suppressor T cells ($CD25^+$Ts) that are able to mediate transplantation tolerance [50] and to arrest the development of autoimmune diabetes [51].

Interest in the role of Ts cells has recently resurged and, among the various populations of Ts cells, naturally occurring thymic and peripheral $CD4^+$ T cells that co-express CD25 are currently the most actively investigated [67]. Although several surface molecules expressed by $CD25^+$Ts cells have been suggested to provide key molecular signals for immunosuppression, multiple mechanisms are probably operative. Based on the essential role of cell-cell contact for suppressive activity *in vitro*, the appropriate localization of $CD25^+$Ts cells could be crucial for their function not only in directing their immunosuppressive activity but also in regulating their homeostasis by guiding them to microenvironmental sources of instruction, survival and/or proliferation signals. CCR4, CCR5 and CCR8, a pattern of chemokine receptors selectively expressed by $CD25^+$Ts cells, could guide them to their cellular targets and control their interaction with APCs and T cells [68].

Two DC subsets, myeloid (M-DCs) and plasmacytoid DCs (P-DCs) have been identified. These subsets are characterized by a distinct expression of pathogen-associated pattern recognition receptors and costimulatory molecules, and by the selective production of immunomodulatory cytokines [28]. We have recently documented that, in contrast to the high production by circulating human M-DCs, the CCR4 ligands CCL17 and CCL22 are poorly produced by P-DCs [69]. It is noteworthy that blood-borne M-DCs, but not P-DCs, constitutively produce CCL17 and CCL22 *ex vivo* [69]. This selective constitutive production of CCR4 ligands by immature M-DCs can lead to the preferential attraction of $CD25^+$Ts cells, ultimately favouring tolerance induction. Intriguingly, the production of the CCR4 ligand CCL22 by M-DCs is markedly enhanced by in vitro treatment with VDR ligands (Table 2). In contrast, immature P-DCs fail to secrete significant amounts of chemokines targeting any of the receptors so far identified on $CD25^+$Ts cells, arguing against a similar function for these cells. Besides maintaining peripheral immunological tolerance in

homeostatic conditions, Ts cells could turn-off and limit ongoing inflammatory responses. Inflammatory signals strongly induce maturation and influx of both M-DCs and P-DCs into secondary lymphoid tissues [28], and maturation of M-DCs and P-DCs enhances their production of several proinflammatory chemokines that can potentially attract different T-cell subsets. Maturing P-DCs, similarly to activated B cells, produce large quantities of the CCR5 ligand CCL4 [69]. Thus, in analogy with the proposed role for CCL4 in $CD25^+$Ts-cell attraction by activated B cells, mature P-DCs could recruit $CD25^+$Ts cells to limit inflammatory responses.

Because DCs are pleiotropic modulators of T-cell activity, pharmacological agents that manipulate DC function to favour the development of Ts cells could be exploited in the treatment of autoimmune diseases and graft rejection [4]. VDR ligands could be ideally suited for this purpose, as shown by their capacity to enhance $CD25^+$Ts cells and promote tolerance induction in transplantation [50] and autoimmune disease [51] models. In both models, treatment with VDR ligands has a profound effect on the migration of effector T cells, preventing their entry into the pancreatic islets [50,51]. It remains to be seen if these agents can also affect the migration of $CD25^+$Ts cells by regulating their chemokine receptor expression or by modulating chemokine production in target tissues such as pancreatic islets. Our preliminary experiments show evidence for the latter possibility.

However, tolerogenic DCs may not always be necessarily involved in the generation of Ts cells by VDR ligands. A combination of $1,25(OH)_2D_3$ and dexamethasone has been shown to induce human and mouse naive $CD4^+$ T cells to differentiate *in vitro* into Ts cells, even in the absence of APCs [66]. These cells produced IL-10, but no IL-5 nor IFN-γ, thus distinguishing them from the previously described Tr1 cells [70]. Upon transfer, the IL-10-producing T cells could prevent central nervous system inflammation, indicating their capacity to exert a suppressive function *in vivo* [66].

III. POSSIBLE MECHANISMS FOR THE IMMUNOMODULATORY EFFECTS OF VDR LIGANDS IN AUTOIMMUNE DISEASE MODELS

The immunoregulatory properties of VDR ligands have been studied in different models of autoimmune diseases (Table 4). Notably, $1,25(OH)_2D_3$ and its analogues can prevent systemic lupus erythematosus in MRL$^{lpr/lpr}$ mice [71-73], experimental allergic encephalomyelitis (EAE) [55,74,75], collagen-induced arthritis [76,77], Lyme arthritis [77], inflammatory bowel disease [78] and autoimmune diabetes in non-obese diabetic (NOD) mice [51,79,80]. $1,25(OH)_2D_3$ analogs are able not only to prevent but also to treat ongoing autoimmune diseases, as demonstrated by their ability to inhibit type 1 diabetes development in adult NOD mice [51] and the recurrence of autoimmune disease after islet transplantation in the NOD mouse [81], or to ameliorate significantly the chronic-relapsing EAE induced in Biozzi mice by spinal cord homogenate [55].

An important property of $1,25(OH)_2D_3$ and its analogs is their capacity to modulate both APCs and T cells. The induction of tolerogenic DCs, which leads to an enhanced number of $CD4^+CD25^+$ regulatory T cells [50,51] renders them appealing for clinical use, especially for the prevention and treatment of autoimmune diseases and grsft rejection. In addition, additive and even synergistic effects have been observed between VDR ligands and immunosuppressive agents, such as CsA and sirolimus, in autoimmune diabetes and EAE models [82,83].

Distinct regulatory mechanisms may predominate in different autoimmune disease models, although a common pattern, characterized by inibition of Th1 cell development, has been frequently observed.

A. Rheumatoid Arthritis

Rheumatoid arthritis (RA) is an immune-mediated disease, with a prominent involvement of Th1 cells [84], characterized by articular inflammation and subsequent tissue damage leading to severe disability

and increased mortality. Among the different animal models of RA, two have been used to test the effects of VDR ligands on the course of the disease, namely Lyme arthritis and collagen-induced arthritis in the mouse. Infection of mice with *Borrelia burgdorferi*, the causative agent of human Lyme arthritis, produces acute arthritic lesions with footpad and ankle swelling. Supplementation with $1,25(OH)_2D_3$ of an adequate diet fed to mice infected with *B. burgdorferi* minimized or prevented these symptoms [77]. The same treatment could also prevent collagen-induced arthritis, and when given to mice with early symptoms prevented the progression to severe arthritis, compared with untreated controls [77]. In a separate study, VDR ligands displayed a similar capacity to prevent and to suppress already established collagen-induced arthritis without inducing hypercalcemia [76].

VDR expression by human articular chondrocytes in osteoarthritic cartilage has been found often associated with sites where matrix metalloproteinases (MMPs) expression was prevalent, in contrast to their virtual absence in normal age-matched cartilage [85]. Together with *in vitro* studies [86], the data suggests that $1,25(OH)_2D_3$ contributes to the regulation of MMPs and PGE_2 production by human articular chondrocytes in osteoarthritic cartilage. Coupled to the evidence obtained in animal models, these results suggest that VDR ligands may be able to control, at least in part, RA development.

B. Type 1 Diabetes

The nonobese diabetic (NOD) mouse, that spontaneously develops type 1 diabetes with a pathogenesis similar to the human disease, represents a useful model for the study of autoimmune diabetes [87]. Several effector mechanisms leading to specific islet β-cell destruction have been identified, including cytotoxic $CD8^+$ lymphocytes and macrophages [88], both of which are regulated by IL-12-dependent T helper 1 (Th1) cells [89]. The activation of Th1 cells specific for β-cell autoantigens could reflect defective

elimination of autoreactive T-cell clones [90], inefficient mechanisms of peripheral tolerance [91], enhanced IL-12 production [92] or impaired suppressive mechanisms [93].

Agents like $1,25(OH)_2D_3$ and its analogs, able to inhibit *in vivo* IL-12 production and Th1 development [55], and to enhance $CD4^+CD25^+$ regulatory T cells [50] may therefore be beneficial in the treatment of type 1 diabetes. $1,25(OH)_2D_3$ itself reduces the incidence of insulitis [94] and prevents type 1 diabetes development [79], but only when administered to NOD mice starting from three weeks of age, before the onset of insulitis. $1,25(OH)_2D_3$ was found ineffective in preventing progression of diabetes in NOD mice when given from 8 weeks of age, when NOD mice present a well established insulitis [95]. However, a combined treatment of 8 week-old NOD mice with the $1,25(OH)_2D_3$ analog MC 1288 and cyclosporine A reduced the incidence of disease, although neither treatment alone was effective [82]. In contrast, we have recently identified the $1,25(OH)_2D_3$ analog 1,25-dihydroxy-16,23Z-diene-26,27-hexafluoro-19-nor vitamin D_3 (Ro 26-2198) that is able, as a monotherapy, to treat the ongoing type 1 diabetes in the adult NOD mouse, effectively blocking the disease course [51]. This property is likely due, at least in part, to the increased metabolic stability of this analog against the inactivating C-24 and C-26 hydroxylations, and the C-3 epimerization [96], resulting in a 100-fold more potent immunosuppressive activity compared to $1,25(OH)_2D_3$. A short treatment with non-hypercalcemic doses of Ro 26-2198 inhibits IL-12 production and pancreatic infiltration of Th1 cells while increasing the frequency of $CD4^+CD25^+$ regulatory T cells in pancreatic lymph nodes, arresting the immunological progression and preventing the clinical onset of type 1 diabetes in the NOD mouse [51].

Protection from type 1 diabetes was found associated with a selective decrease of Th1 cells in the pancreatic lymph nodes and in the pancreas, without a marked deviation to the Th2 phenotype. The frequency of $CD4^+CD25^+$ cells in the pancreatic lymph nodes of Ro 26-2198-treated NOD mice was two-fold higher compared to untreated 8 week-old and to age-matched vehicle-treated controls. These

cells were anergic, as demonstrated by their impaired capacity to proliferate and secrete IFN-γ in response to TCR ligation, inhibited the T cell response to the pancreatic autoantigen IA-2, and delayed disease transfer by pathogenic $CD4^+CD25^-$ cells [51].

Immature DCs have been shown to induce $CD4^+$ cells with regulatory properties [31], and arrest of DCs at the immature stage induced by Ro 26-2198 treatment could account for the enhanced frequency of $CD4^+CD25^+$ cells. $CD4^+CD25^+$ regulatory T cells appear to play an important role in controlling the progression of type 1 diabetes in NOD mice, because a low level of $CD4^+CD25^+$ T cells correlates with exacerbation and acceleration of the disease [93]. It is likely that this cell population is more relevant than Th2 cells in disease control, although both could contribute to protection. Indeed, $1,25(OH)_2D_3$ can induce regulatory cells with disease-suppressive activity in the NOD mouse [79] and a disease-preventing $1,25(OH)_2D_3$ analog could deviate pancreas-infiltrating cells to the Th2 phenotype [82]. In addition, the pro-apoptotic activity of $1,25(OH)_2D_3$ and its analogs can restore the defective sensitivity to apoptosis of NOD lymphocytes [97], leading to a more efficient elimination of potentially dangerous autoimmune effector cells. The increased apoptosis induced by $1,25(OH)_2D_3$ and its analogs in DCs [39] and T cells [97] has been observed after different apoptosis-inducing signals, and could help to explain why short-term treatments with these agents afford long-term protection and promote tolerance induction.

The observation that ongoing type 1 diabetes in the adult NOD mouse can be arrested by a relatively short course of treatment with a $1,25(OH)_2D_3$ analog [51] suggests that a similar treatment may also inhibit disease progression in prediabetic or newly diagnosed type 1 diabetes patients. Polymorphisms of the vitamin D receptor gene have been associated with type 1 diabetes in different populations [98,99], and epidemiological studies have shown a higher incidence of the disease in northern than in southern latitudes [100], suggesting a possible involvement of a $1,25(OH)_2D_3$ deficiency in the pathogenesis of type 1 diabetes. This is further supported by a large population-based case-control study [101] and by a birth-cohort study [102]

showing that the dietary vitamin D supplementation contributes to a significantly decreased risk of type 1 diabetes development.

C. Experimental Allergic Encephalomyelitis

Experimental allergic encephalomyelitis (EAE) is considered as a model for multiple sclerosis (MS), and in both diseases Th1-type cells specific for myelin antigens appear to play a pathogenic role [4]. $1,25(OH)_2D_3$ and the non-hypercalcaemic analogue (5Z,7E,23E,24aE)-(1S,3R)-24a,24b-dihomo-9,10-seco-cholesta-5,7,10(19),23,24a pentaene-1,3,25-triol (Ro 63-2023) have been shown to be selective and potent inhibitors of Th1 development *in vitro* and *in vivo* without inducing a deviation to the Th2 phenotype [55]. Administration of $1,25(OH)_2D_3$ or its analogue could prevent chronic-relapsing experimental allergic encephalomyelitis (CR-EAE) induced by the MOG peptide 35-55 in Biozzi AB/H mice, and this was associated with a profound reduction of MOG_{35-55}-specific proliferation and Th1 cell development. Importantly, the non-hypercalcaemic analogue Ro 63-2023 also provided long-term protection from EAE relapses induced by immunization with spinal cord homogenate when administered for a short time either at symptom onset or even after the first peak of disease. Neuropathological analysis showed a significant reduction of inflammatory infiltrates, demyelinated areas and axonal loss in brains and spinal cords of treated mice. Thus, inhibition of IL-12-dependent Th1 cell development is associated with effective treatment of CR-EAE, further suggesting the feasibility of this approach in the treatment of multiple sclerosis [4].

These results demonstrate a correlation between the capacity of $1,25(OH)_2D_3$ and the less calcemic analog Ro 63-2023 to inhibit IL-12-dependent Th1 development and to treat EAE, a correlation that was not established by previous studies [59,74,75,103]. Conversely, a systemic increase in the transcripts for TGF-ß1 and IL-4 was suggested to be responsible for the capacity of $1,25(OH)_2D_3$ to inhibit EAE [59], in

contrast with the results of Mattner et al. [55], demonstrating that $1,25(OH)_2D_3$ is a potent inhibitor of Th1 development and EAE without deviating the response to the Th2 pathway, as well as with the preferential inhibition of Th1 responses by $1,25(OH)_2D_3$ observed by Lemire et al. [36]. The reasons for this discrepancy are not clear, although the different EAE models analyzed could play a role. TGF-β1 [104] and IL-4 [105,106] have been reported to be beneficial in EAE but this activity has been ascribed to indirect inhibition of encephalitogenic Th1 cells. IL-10 also appears to be critical in the control of pathogenic Th1 responses in EAE [107], and $1,25(OH)_2D_3$ has been shown *in vitro* to strongly enhance IL-10 production by human DCs [39] and to favour the induction of IL-10-producing regulatory T cells [66].

$1,25(OH)_2D_3$ can cross the intact blood-brain barrier [108] and could therefore directly inhibit CNS APCs, like microglia, that regulate intracerebral T cell responses [109], or target infiltrating T cells as well as recruited APCs. $1,25(OH)_2D_3$ administration inhibits the expression of inducible nitric oxide synthase in macrophages, activated microglia and astrocytes during EAE [110], and this could also contribute to amelioration of the disease. Alternatively, the immunomodulatory effects of VDR ligands could be mainly exerted in peripheral lymphoid organs leading to inhibition of encephalitogenic T cell development.

D. Inflammatory Bowel Disease

Inflammatory bowel diseases (IBDs) are immune-mediated diseases of unknown aetiology affecting the gastrointestinal tract. At least two distinct forms of IBDs have been defined, ulcerative colitis and Crohn's disease. These are chronic recurring illnesses most commonly involving inflammation of the terminal ileum and colon, although they can also affect many sites throughout the alimentary tract. In addition to genetic factors, including also VDR gene polymorphisms [111], the environment contributes to IBD development, and vitamin D may be an important environmental component in this respect. Lower amounts of $1,25(OH)_2D_3$ are synthesized from sunlight exposure in areas in which IBDs occur most often, such as

North America and Northern Europe [112], a situation common to other autoimmune diseases [113], in particular type 1 diabetes [100] and multiple sclerosis [114]. Dietary intake of vitamin D is problematic because few foods are naturally rich in vitamin D and weight loss, with consequently reduced vitamin D intake, occurs in the majority of IBD patients.

In IBD models, the immune-mediated attack against the gastro-intestinal tract has been shown to be mediated by Th1 cells [115], and the production of Th1-type cytokines has also been found associated with human IBDs [116]. Animal models have been developed in which IBD symptoms occur spontaneously, and a well-studied one is the IL-10 knock-out (KO) mouse [117]. In conventional animal facilities, IL-10 KO mice develop enterocolitis within 5 to 8 weeks of life, and approximately 30% of these mice die of severe anemia and weight loss [117]. The enterocolitis that develops in IL-10 KO mice is due to an uncontrolled immune response to conventional microflora, because germfree IL-10 KO mice do not develop disease, and mice raised in specific pathogen–free facilities develop a milder disease [117]. IL-10 KO mice were made vitamin D deficient, vitamin D sufficient or supplemented with $1,25(OH)_2D_3$ [78]. Vitamin D-deficient, in contrast to vitamin D-sufficient IL-10 KO mice, rapidly developed diarrhea and a severe wasting disease. Administration of $1,25(OH)_2D_3$ significantly ameliorated IBD symptoms in IL-10 KO mice and treatment for as little as 2 weeks blocked the progression and ameliorated symptoms in mice with already established IBD [78]. This would be consistent with the observation that patients with Crohn's disease have depressed IL-10 production and respond positively to IL-10 administration [118]. Interestingly, VDR ligands inhibit the proliferation of rectal epithelial cells [119] and of T cells [120] in active ulcerative colitis patients, further suggesting their possible use in the treatment of IBDs.

E. Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a T-cell dependent antibody-mediated autoimmune disease and the mouse strain MRL$^{lpr/lpr}$ spontaneously develops a SLE-like syndrome sharing many immunological features with human SLE. Administration of VDR ligands significantly prolonged the average life span of MRL$^{lpr/lpr}$ mice and induced a significant reduction in proteinuria, renal arteritis, granuloma formation and knee joint arthritis [71-73]. In addition, dermatological lesions, like alopecia, necrosis of the ear, and scab formation, were also completely inhibited by 1,25$(OH)_2D_3$ therapy [73].

These data suggest a beneficial role of VDR ligands in the treatment of human SLE. Indeed, VDR ligands can significantly reduce cell proliferation and IgG production, both polyclonal and anti-dsDNA, while enhancing B cell apoptosis in lymphocytes from SLE patients [121]. However, it has also been shown that in (NZBxW)F1 mice, prone to developing SLE, treatment with 1,25$(OH)_2D_3$ worsens the disease, possibly explaining how sunlight could be a factor aggravating the course of SLE [122]. These results could be reconciled by the observation that MRL$^{lpr/lpr}$ mice receiving 1,25$(OH)_2D_3$ and a diet with a normal/high calcium content (0.87%) showed reduced SLE, whereas the same treatment in MRL$^{lpr/lpr}$ mice on a very low calcium content diet (0.02%) led to accelerated and more severe SLE [7], a situation already noted in EAE [123].

F. Psoriasis

Psoriasis is a chronic inflammatory skin disease that affects about 2% of the population. Although the pathogenesis of psoriasis is still incompletely understood, it appears to be primarily a Th1-mediated autoimmune disease involving hyperproliferation of keratinocytes [124]. Given the capacity of VDR ligands to modulate both cell types, their success in treating psoriasis is perhaps not surprising. VDR ligands are currently the mainstay treatment in mild and moderate psoriasis, accounting for about 50% of all drugs used

to treat this disease. At present, VDR ligands are used only topically, because a safe analog for systemic use has not yet been developed. In addition to topical calcitriol, calcipotriol and tacalcitol have shown efficacy and safety in extensive controlled studies [125-127].

Mechanistically, the beneficial effects of VDR ligands in psoriasis could reflect inhibition of proliferation and cytokine production by skin-infiltrating T cells [128]. VDR ligands have been shown to increase IL-10 production in psoriatic lesions [129] and to decrease IL-6 and IL-8 secretion by keratinocytes [130]. In addition, $1,25(OH)_2D_3$ but not IL-10 could prevent leukocyte activation and reduce the histological and clinical scores in human psoriatic skin transplanted on to SCID mice [131]. The apoptotic process in psoriatic lesions has been suggested to be in part regulated by Bcl-xL, and decreasing the expression of Bcl-xL by treatment with VDR ligands might ameliorate psoriatic lesions by contributing to the completion of the apoptotic process [132].

IV. BENEFICIAL EFFECTS OF VDR LIGANDS IN ALLOGRAFT REJECTION

Acute allograft rejection is mediated by immunological mechanisms, with APCs, in particular dendritic cells (DCs) and T cells playing a major role, whereas chronic rejection is mediated by a poorly understood combination of immunological and non immunological mechanisms [133]. Current immunosuppressive treatments based on small molecules (cyclosporine A, tacrolimus, sirolimus, mycophenolate mofetil) or on biologicals (anti-CD3, anti-CD52, anti-IL2R) can target quite effectively both T lymphocytes and APCs, but they have also important side effects. Drugs targeting both cell subsets, but devoid of major side effects, would therefore represent a useful addition to the available immunosuppressive agents. In addition, while near optimal control of acute rejection and adequate short-term graft survival have been achieved, problems associated with chronic rejection and long-term

immunosuppressive management are rising [134]. Agents able to inhibit chronic rejection, and potentially able to promote transplantation tolerance, would thus fill an important unmet medical need.

VDR ligands have pleiotropic immunoregulatory activities that are able to control allograft rejection, as demonstrated in different models of experimental organ transplantation, both acute and chronic (Table 5). APCs and T cells can be direct targets of the hormone, leading to the inhibition of pathogenic effector T cells and enhancing the frequency of T cells with suppressive properties, largely via induction of tolerogenic DCs. These immunoregulatory activities, coupled with the absence of major side effects once calcemia is under control, have been translated into effective immunointervention in a variety of graft rejection models, both acute and chronic, showing potential for clinical applications [135,136].

A. Inhibition of Acute Rejection

$1,25(OH)_2D_3$ and its analogues can significantly prolong allograft survival in heart [137-139], kidney [140,141], liver [142], pancreatic islets [50,97,143,144], skin [145] and small bowel allografts [138]. In general, these effects have been achieved at the maximum tolerated dose, without inducing hypercalcemia, the major side effect of treatment with VDR ligands. In most experimental models, the acute rejection has been further delayed by combining VDR ligands with a suboptimal dose of CsA or other immunosuppressive agents (Table 5). Although treatment with VDR ligands has consistently shown efficacy in delaying the acute allograft rejection, the effects on chronic rejection are probably the most interesting.

B. Inhibition of Chronic Rejection

VDR ligands can inhibit, in association with low doses of Cyclosporin A (CsA), not only acute but also chronic allograft rejection, as documented by inhibition of adventitial inflammation and intimal hyperplasia in rat aortic allografts [146]. While the prevention of leukocyte infiltration into the adventitia is

probably due to the immunomodulatory properties of VDR analogs, the inhibition of intimal cell proliferation, both endothelial and smooth muscle cells, is likely induced by their capacity to regulate cell growth. The $1,25(OH)_2D_3$ analogue MC 1288 also reduced clinical and histological signs of chronic graft rejection in rat kidney allografts [140]. The chronic allograft damage index, reflecting the sum of interstitial inflammation and fibrosis, glomerular mesangial matrix increase and sclerosis, vascular intimal proliferation and tubular atrophy, was significantly reduced in recipients treated with MC 1288 and CsA compared to CsA only. Renal graft loss has been found decelerated, in a retrospective study, in patients treated with $1,25(OH)_2D_3$ [147], further suggesting its clinical applicability to inhibit chronic graft rejection.

C. Immunoregulatory Mechanisms Inhibiting Graft Rejection

The induction of tolerogenic DCs by VDR ligands, which leads to an enhanced number of $CD4^+CD25^+$ regulatory T cells *in vivo* [50,51] are likely to play an important role in controlling graft rejection, both acute and chronic, and in favoring the establishment of transplantation tolerance. A short treatment with $1,25(OH)_2D_3$ and mycophenolate mofetil, a selective inhibitor of T and B cell proliferation [148] that also modulates APCs [149], induces tolerance to islet allografts associated with an increased frequency of $CD4^+CD25^+$ regulatory T cells able to adoptively transfer transplantation tolerance [50]. The induction of tolerogenic DCs could indeed represent a therapeutic strategy promoting tolerance to allografts [150] and the observation that immature myeloid DCs can induce T cell tolerance to specific antigens in human volunteers represents an important proof of concept for this approach [151].

The direct effects of VDR ligands on T cells, in particular the inhibition of IL-2 and IFN-γ production, could also play a role in inhibiting graft rejection. $1,25(OH)_2D_3$ inhibits IL-2 secretion by impairing the formation of the transcription factor complex NF-AT [56,57], and IFN-γ through interaction of the ligand-bound VDR complex with a VDRE in the promoter region of the cytokine [58]. In addition, a

combination of $1,25(OH)_2D_3$ and low-dose CsA inhibited the expression of IL-2 and IL-12, and increased significantly IL-10 expression levels in kidney allografts [141]. It is also possible, as suggested by our preliminary experiments, that VDR ligands may inhibit the production of proinflammatory chemokines by cells of the transplanted organ, thus inhibiting leukocyte recruitment to the graft. Additional mechanisms could rely on the capacity of $1,25(OH)_2D_3$ to significantly reduce bioactive renal TGF-β1 by interacting with Smad proteins, important regulators of TGF-β signal transduction [152]. Since TGF-β has a pronounced pro-fibrotic activity, its decrease in the kidney tissue may inhibit the evolution of chronic rejection in kidney transplants.

D. Combination of VDR Ligands with Immunosuppressive Agents

Based on the available evidence of a pro-tolerogenic effect and a reduced incidence of chronic rejection, VDR ligands could be added to standard immunosuppressive regimens in the treatment of allograft rejection. Additive and even synergistic effects have been observed between $1,25(OH)_2D_3$ or its analogues and immunosuppressive agents, in particular CsA, tacrolimus and sirolimus [83]. These effects have been confirmed in models of graft rejection (Table 5), making VDR ligands potentially interesting as dose-reducing agents for conventional immunosuppressive drugs in clinical transplantation.

Another positive feature of adding VDR ligands to standard immunosuppressive regimens is their protective effect on bone loss. A rapid bone loss is usually seen after organ transplantation and is enhanced by some immunosuppressive regimens, in particular those based on tacrolimus and steroids [153]. $1,25(OH)_2D_3$ administration has been shown to prevent bone loss in transplanted patients [154-156], although standard prophylactic measures may not always be sufficient to prevent loss of bone mass [157], and $1,25(OH)_2D_3$ analogues with a wider therapeutic window could serve also this function. In addition, 22-oxa-$1,25(OH)_2D_3$ (OCT) has been shown to exert an anabolic effect on bone reconstruction by

vascularized bone allografts in rats [158], indicating a specific advantage of VDR ligand administration in bone transplantation. Importantly, the use of VDR ligands to control allograft rejection does not appear to increase opportunistic infections [159], a major side effect induced by anti-rejection drugs, in particular calcineurin inhibitors and glucocorticoids.

V. Conclusions

VDR ligands, in addition to controlling calcium metabolism and exerting important effects on the growth and differentiation of many cell types, possess pronounced pro-tolerogenic immunoregulatory properties. VDR ligands can act directly on T cells, but DCs appear to be primary targets for their tolerogenic properties. The capacity of VDR ligands to target DCs and T cells is mediated by VDR expression in both cell types and by the presence of common targets in their signal transduction pathways, such as the nuclear factor NF-*k*B that is down-regulated in APCs and in T cells. VDR ligands can induce *in vitro* and *in vivo* tolerogenic DCs able to enhance $CD4^+CD25^+$ suppressor T cells that, in turn, inhibit Th1 cell responses.

These mechanisms of action can explain some of the immunoregulatory properties of VDR ligands that are potentially relevant for the treatment of Th1-mediated autoimmune diseases and allograft rejection, but may also represent a physiologic element in the regulation of innate and adaptive immune responses (Fig. 2). A challenge for the future is the development of safe VDR ligands for the systemic treatment of psoriasis, and the translation of orally active VDR ligands to the treatment of other autoimmune diseases and allograft rejection. The accumulating evidence for the multiple immunomodulatory mechanisms regulated by VDR ligands indeed represents a sound basis for a further exploration of their potential in the development of therapies for several immuno-mediated disorders.

References

EN.REFLIST

Figure legends

Figure 1. Dendritic cells play a key role in the generation of effector and regulatory T cells. DCs expressing high levels of surface costimulatory molecules, e.g. CD40, CD80, CD86, and secreting IL-12, induce effector T cells, notably Th1 cells. Conversely, DCs expressing low levels of costimulatory molecules, secreting IL-10, and expressing high levels of inhibitory molecules (e.g. ILT3) favour the induction and/or the enhancement of regulatory/suppressor T cells.

Figure 2. Mechanisms involved in the regulation of immune responses by VDR ligands. VDR ligands (VDRL) can modulate the immune response via several mechanisms in secondary lymphoid organs and in target tissues. In secondary lymphoid organs, $1,25(OH)_2D_3$ inhibits IL-12 production and down-regulates costimulatory molecule expression (CD40, CD80, CD86) expressed by dendritic cells (DCs), while upregulating their IL-10 production, thus inhibiting the development of Th1 cells and favouring the induction of $CD4^+CD25^+$ regulatory T cells and of Th2 cells. VDR ligands can also inhibit the migration of Th1 cells, and they upregulate ILT3 expression and CCL22 production by myeloid DCs (M-DC), enhancing the recruitment of $CD4^+CD25^+$ regulatory T cells and of Th2 cells. In addition, VDR ligands exert direct effects on T cells by inhibiting IL-2 and IFN-γ production. Macrophages (MΦ), as well as DCs and T cells, can synthesize $1,25(OH)_2D_3$ and this may also contribute to the regulation of the local immune response. In target tissues, pathogenic Th1 cells, that can damage target cells via induction of cytotoxic T cells (CTL) and activated macrophages, are reduced in number and their activity is further inhibited by $CD4^+CD25^+$ regulatory T cells and by Th2 cells induced by VDR ligands. Black arrows indicate stimulation, blunted red arrows inhibition, and broken arrows cytotoxicity.

Table 1. Key attack points for selective immunomodulation

•**Targeting the MHC/TCR complex of pathogenic T cells**

- MHC blockade, Deletion, Altered Peptide Ligands

- Co-receptors (CD4)

•**Costimulation blockade**

- Inhibition of interaction between CD28-CD80/CD86; CD154-CD40, LIGHT-HVEM; ICOS-ICOSL; CD134-CD134L - Upregulation of negative co-regulators (CD152, PD-1)

•**Immune deviation**

- Skewing to Th2 via APC or direct T-cell modulation•**Cytokine-based immunointervention**

- Inhibition of pro-inflammatory cytokines (IL-1, IL-2, IFN-γ, IL-12, TNF-α)

- Administration of anti-inflammatory cytokines (IL-4, IL-10, IFN-β, TGF-β)

•**Induction of regulatory T cells**

- T cell/TCR peptide vaccination, APC manipulation, cytokines (IL-10, TGF-β)

•**Targeting leukocyte trafficking**

- Adhesion molecules, chemokines, chemokine receptorsTable 2. Phenotypic and functional modifications induced by VDR ligands in human myeloid dendritic cells

PHENOTYPE	EFFECT
Maturation marker expression	
CD83	decreased
DC-LAMP	decreased
Antigen uptake	
Mannose receptor expression	increased
Costimulatory molecule expression	
CD40	decreased
CD80	decreased
CD86	decreased
Inhibitory molecule expression	
ILT3	increased
ILT4	unmodified
B7-H1	unmodified
Chemokine receptor expression	
CCR7	decreased

FUNCTION	EFFECT
Cytokine production	
IL-10	increased
IL-12	decreased
Chemokine production	
CCL2	increased
CCL17	decreased

CCL18	increased
CCL20	decreased
CCL22	increased
Apoptosis	
Maturation-induced	increased
T-cell activation	
Response to alloantigens	decreased

Compiled from refs. [39,160] and from the author's unpublished data.

Table 3. Effects of VDR ligands on T cells

Effect	**References**
Inhibition of T cell proliferation	18
Induction of hyporesponsiveness to allo and self antigens	39-43
Inhibition of IL-2 production	56,57
Inhibition of IFN-γ production	58,65
Inhibition of Th1 cell development	36,55
Variable effects on IL-4 production and deviation to Th2	52,55,59,63-65
Increased production of IL-10	66
Increased expression of CD152	39,50
Down-regulation of CD95 expression	60
Enhanced frequency of regulatory T cells	50,51,66

Table 4. Effects of VDR ligand treatment in animal models of autoimmune diseases

Experimental models	**Main effects**	**References**
Arthritis	Decreased incidence and severity of collagen-induced or Lyme arthritis, also when given at disease onset	76,77
Autoimmune diabetes	Inhibition of insulitis and reduction of diabetes, even when given after islet infiltration	79,80,82,161
Experimental allergic encephalomyelitis	Prevention and treatment of disease, inhibition of relapses	52,55,74,103
Inflammatory bowel disease	Significant amelioration of symptoms, block of of disease progression	78
Psoriasis	Inhibition of leukocyte activation and amelioration of histological and clinical sign of disease in human psoriatic skin grafts transplanted to SCID mice	131
Systemic lupus erythematosus	Inhibition of proteinuria, prevention of skin lesions	71,73

Table 5**.** Effects of VDR ligand treatment in animal models of transplantation

Transplantated organ	**VDR ligand tested**	**Synopsis of experimental protocol**	**Main effects**	**References**
Aorta	MC 1288	DA aortic allografts into WF rats treated with MC 1288 (0.1 μg/kg/eod) alone or together with CsA (5 mg/kg/d)	Reduced proliferation and activation of adventitial T cells induced by MC 1288 alone, decreased intimal hyperplasia when co-administered with CsA	[146]
Bone marrow	MC 1288	Lewis bone marrow into BN rats treated with MC 1288 (0.1 μg/kg/eod) alone or together with CsA (5 mg/kg/d)	Decreased clinical and histological signs of graft-versus-host disease induced by MC 1288, decreased intimal hyperplasia when co-administered with CsA	[162]
Heart	$1,25(OH)_2D_3$1 6-ene-$1,25(OH)_2D_3$ Paricalcitol MC 1288	Non vascularized and vascularized heart allografts in mice or rats. Recipients were treated with VDR ligands alone or combined with low doses of CsA	All VDR ligands induced marked prolongation of heart allografts, usually superior to that induced by a full dose of CsA	[137-139]

Kidney	$1,25(OH)_2D_3$ MC 1288	Kidney grafts in high responder rat strain combinations. Recipients were treated with VDR ligands alone or combined with low doses of CsA	$1,25(OH)_2D_3$ significantly prolonged allograft survival, preserved renal creatinine clearance and decreased proteinuria. In combination with CsA induced inhibition of IL-2 and IL-12 as well as significant upregulation of IL-10 expression. MC 1288 alone reduced clinical and histological signs of chronic rejection, and combined with CsA reduced the number of acute rejection episodes.	[140,141]
Liver	$1,25(OH)_2D_3$	ACI vascularized liver into Lewis rats treated with $1,25(OH)_2D_3$ (0.1 or 1 μg/kg/d) alone or together with CsA (2 mg/kg/d)	Prolonged graft survival by decreasing the severity of acute rejection	[142]

Pancreatic islets	$1,25(OH)_2D_3$ KH 1060 MC 1288 TX 527	B6 islets transplanted into diabetic BALB/c mice; syngeneic or xenogeneic islets transplanted into NOD mice. Recipients were treated with VDR ligands alone or combined with mycophenolate mofetil, CsA, or IFN-β	Prevention of allogeneic graft rejection and induction of transferable tolerance; prevention of autoimmune diabetes recurrence	[50,81,143,144]
Skin	$1,25(OH)_2D_3$ KH 1060 CB 966	B6 skin transplanted into CBA mice treated with VD ligands alone or combined with CsA	Significantly prolonged graft survival, most evident with KH 1060. Additive effects when combined with CsA	[145]
Small bowel	MC 1288	Small bowel transplanted into allogenic rats treated with MC 1288 (0.1 μg/kg/d)	Reduced amounts of hyaluronan secreted into the intestina lumen	[138]